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Lethal and Sublethal Exposure and Recovery Effects of Ozone-Produced Oxidants on Adult White Perch (Morone americana Gmelin)

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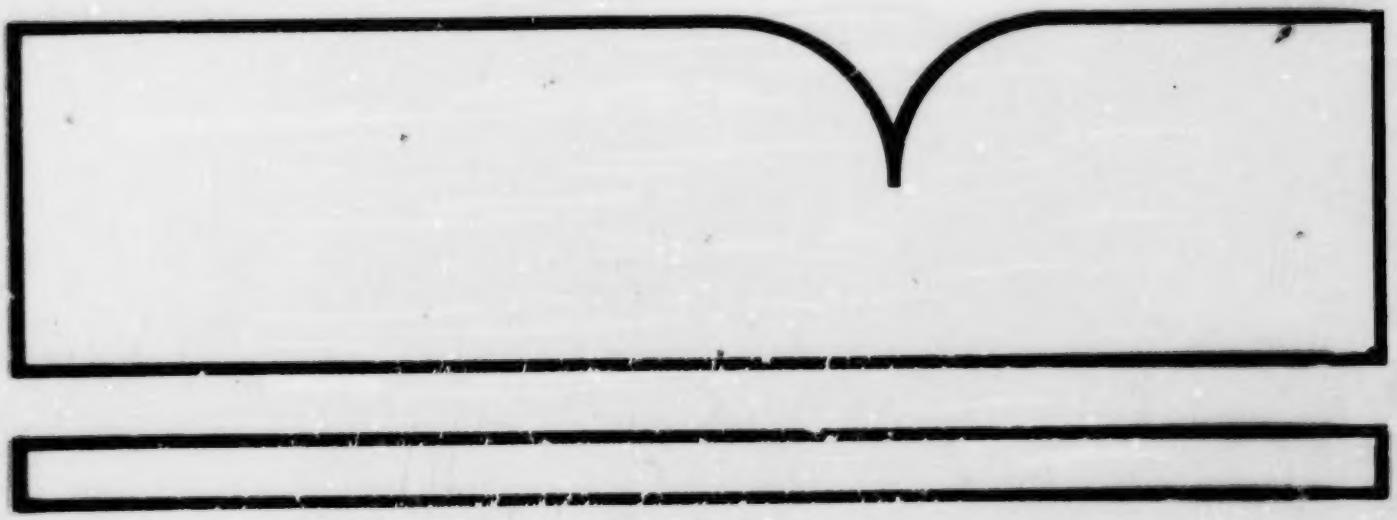

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16. ABSTRACT

Adult white perch (Morone americana), acclimated to 15°C, were exposed to a series of ozone-produced oxidant (OPO) concentrations for 96 h using continuous flow bioassay techniques. Toxicity were analyzed using both responses surface modeling and standard probit regression. White perch were also exposed to a series of near and sublethal OPO concentrations, selected from the acute toxicity study, fo 96 h and then placed in clean non-ozonated water for 14 days. Blood pH, hematocrit and gill histopathology were analyzed during exposure at 24, 48 and 96 h and after 4 and 14 days in the recovery period. Blood pH and hematocrit levels were analyzed statistically using standard ANOVA and multiple range tests. Histopathological effects were examined using both light microscopy and scanning electron microscopy. The 24-, 48- and 96-h LC_{50}'s were 0.38, 0.26 and 0.20 mg OPO^{-1}, respectively. Blood pH was significantly reduced at concentrations >0.15 mg $OPO1^{-1}$ but not at 0.10 mgl^{-1} or lower concentrations. Hematocrit significantly increased at concentrations >0.10 mg $OPO1^{-1}$. Histipathological examination revealed minimal effects on gill tissue at 0.01 mg $OPO1^{-1}$, moderate epithelial sloughing and heavy, mucus production at 0.05 mg $OPO1^{-1}$ and extreme tissue damage at concentrations >0.10 mg 1^{-1}.

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LETHAL AND SUBLETHAL EXPOSURE AND RECOVERY EFFECTS OF OZONE-PRODUCED OXIDANTS ON ADULT WHITE PERCH (*MORONE AMERICANA* GMELIN)

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Abstract—Adult white perch (*Morone americana*), acclimated to 15 C, were exposed to a series of ozone-produced oxidant (OPO) concentrations for 96 h using continuous flow bioassay techniques. Toxicity data were analyzed using both response surface modeling and standard probit regression. White perch were also exposed to a series of near and sublethal OPO concentrations, selected from the acute toxicity study, for 96 h and then placed in clean non-ozonated water for 14 days. Blood pH, hematocrit and gill histopathology were analyzed during exposure at 24, 48 and 96 h and after 4 and 14 days in the recovery period. Blood pH and hematocrit levels were analyzed statistically using standard ANOVA and multiple range tests. Histopathological effects were examined using both light microscopy and scanning electron microscopy. The 24-, 48- and 96-h LC_{50}'s were 0.38, 0.26 and 0.20 mg OPO l^{-1}, respectively. Blood pH was significantly reduced at concentrations $\geq$0.15 mg OPO l^{-1} but not at 0.10 mg l^{-1} or lower concentrations. Hematocrit significantly increased at concentrations $\geq$0.10 mg OPO l^{-1}. Histopathological examination revealed minimal effects on gill tissue at 0.01 mg OPO l^{-1}, moderate epithelial sloughing and heavy mucus production at 0.05 mg OPO l^{-1} and extreme tissue damage at concentrations $\geq$0.10 mg l^{-1}. Results from both the acute toxicity and the exposure and recovery study were compared with the effects of chlorine-produced oxidants (CPO) obtained from the literature. Both OPO and CPO appear to have similar effects on adult white perch.

INTRODUCTION

Chlorine has been widely used for disinfection of municipal and industrial wastewaters and the control of biological fouling in power stations. Several recent studies have shown, however, that chlorine and its residual by-products are toxic to aquatic organisms (Tsai, 1975; Whitehouse, 1975; Brungs, 1976; Davis & Middaugh, 1976; Morgan & Carpenter, 1978; Hall *et al.*, 1981, 1982) and may also produce reaction products hazardous to man (Jolley, 1973; Glase *et al.*, 1976; Jolley *et al.*, 1978, 1980). For these reasons, several alternatives have been proposed for chlorine (U.S. Environmental Protection Agency, 1976; Burton & Liden, 1978; Garey, 1980). One of the most viable alternatives to chlorine for water and wastewater treatment is ozone (Evans, 1972; Blogoslowski & Rice, 1975; Fochtman *et al.*, 1977).

Investigations on the toxicity of ozone to estuarine and marine fish are limited (Toner & Brooks, 1975; Block *et al.*, 1978). Furthermore, it has been shown that persistent oxidative by-products occur in sea water after ozonation which do not occur in fresh water (Blogoslawski *et al.*, 1976; Crecelius, 1979; Kosak-Channing & Helz, 1979; Stewart *et al.*, 1979). It is important, therefore, that more research be conducted on the toxicity of ozone in saline waters.

This study was initiated to evaluate the effects of ozone-produced oxidants (OPO) on adult white perch (*Morone americana*) in estuarine water. This study was designed to: (1) establish acute dose-mortality relationships; (2) elucidate the physiological basis of acute toxicity; (3) determine the possible recovery potential of fish exposed to sublethal concentrations of OPO which may move to non-ozonated water; and (4) compare the effects of OPO to those of previously published chlorine toxicity data.

The terms ozone-produced oxidant (OPO) and chlorine-produced oxidant (CPO) are used throughout this paper to refer to the amperometrically detectable total resident oxidant concentrations (measured as total residual chlorine) produced in solution following ozonation or chlorination, respectively. Thus, the term CPO is synonymous with the more commonly used terms total residual chlorine (TRC) and total residual oxidant (TRO). The term "dose", as used in this paper, is defined as the product of con-

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centration × exposure time and does not imply that a known quantity of toxicant has been directly administered to the organism.

MATERIALS AND METHODS

White perch were collected by beach seine or trawl from the Patuxent River near Benedict, Maryland when ambient temperatures approximated the desired experimental temperature of 15°C. Perch were acclimated for at least 10 days in 1900 or 3800 l. circular fiberglass holding tanks supplied with a continuous flow of unfiltered Patuxent River water before experimentation. Fish were fed once daily during acclimation with frozen shrimp or fresh clams and maintained under a 12 h light:12 h dark photoperiod. The white perch used in these studies had a mean (±SD) total length, standard length and total wet weight of 164.7 (±16.61) mm, 135.9 (±12.67) mm and 54.1 (±15.27) g, respectively.

Ozone gas was produced with an electrical discharge type ozonator (Model T-816, Welsbach Ozone Systems Corp., Philadelphia, PA) using dry aviator oxygen. The ozone–oxygen mixture (≃4% O_3 by volume) was bubbled into the base of an 11.5-l., polycarbonate counter current contact column (7 cm i.d.; 2.75 m high) at 1.5 l min^{-1} through a 3-cm fritted glass disc. Filtered (5 μ) Patuxent River water was pumped into the top of the column while ozonated water was drawn from the bottom and fed to glass rotometers through which it was metered into the experimental apparatus.

Filtered water from a constant pressure head tank was continuously delivered to the exposure tanks through ≃2 cm i.d. CPVC tubes. These delivery tubes were fitted with "tees", through which ozonated water from the rotometers could be injected. No oxidant was injected into the delivery tube which supplied the control tank.

Ozone-produced oxidant (OPO) concentrations were measured amperometrically as equivalent total residual chlorine (TRC) (APHA, 1976) using a PAR polarographic analyzer (Model 174A; Princeton Applied Research Corp., Princeton, NJ) equipped with a 10-g, rotating spiral platinum electrode and a standard platinum disc reference electrode. Phenylarsine oxide (2.82×10^{-3} N) was used as the titrant and was delivered with a syringe microburet (Model SB2; Micro-Metric Instrument Co., Cleveland, OH) capable of titrant additions, as small as 3 μl. The potassium iodide and pH 4 reagents were prepared according to *Standard Methods* (APHA, 1976) and were premixed in the bottom of a 200-ml volumetric sample collection flask to minimize possible underestimation of the oxidant concentration (Carpenter *et al.*, 1977; Crecelius *et al.*, 1978).

Toxicity studies

Acute toxicity studies were conducted to define dose-mortality relationships for adult white perch exposed to a series of OPO concentrations. Ten fish were exposed to each concentration (±SD) of 0.000, 0.064 (±0.007), 0.094 (±0.019), 0.161 (±0.011), 0.216 (±0.015), 0.259 (±0.016), 0.298 (±0.013), 0.301 (±0.016), 0.331 (±0.014), 0.335 (±0.011), 0.391 (±0.014) and 0.424 (±0.018) mg OPO l^{-1} (as equivalent TRC) for 96 h at 15°C. Fish were exposed to the various test concentrations in 45 l., all-glass aquaria; each supplied with a continuous flow of ≃2 l min^{-1} of the appropriate test solution. Mean water quality (±SD) during the acute study was: temperature 15.0 (±0.07) C, salinity 12.7 (±0.32) ‰, pH 8.0 (±0.03) and dissolved oxygen concentrations which ranged from 11 to 17 mg l^{-1} depending upon the OPO concentration. Cessation of opercular movement for 1 min was used as the criterion for death.

Toxicity data were statistically analyzed using response surface modeling techniques. The following linear model was initially introduced to the stepwise regression procedure of SAS (SAS Institute Inc., 1979):

$$\text{Arcsine}\,[(r/n)^{0.5}] = a + b(C) + c(T) + d(D) + e(C^2) + f(T^2) + g(DC) + h(DT) + i(D^2) + j(\log C) + k(\log T)$$

where

C = OPO exposure concentration (mg l^{-1})
T = exposure time (h)
D = dose ($C \times T$)
r = mortality response at a given dose
n = sample size at a given dose
a–k = coefficients estimated by the least squares procedure.

Only those variables making a significant ($P < 0.05$) contribution to the final model were selected. In addition to the response surface model a SAS probit analysis (SAS Institute Inc., 1979) was used to calculate standard LC_{50} values to facilitate comparisons with the literature.

Exposure and recovery studies

Near- and sublethal OPO concentrations were selected for the sublethal studies based upon the results of the acute toxicity study. Thirty adult white perch were exposed to each OPO concentration (±SD) of 0.000, 0.011 (±0.002), 0.051 (±0.010), 0.101 (±0.040) and 0.152 (±0.045) mg l^{-1} for 96 h. At the end of this exposure period injection of ozonated water was stopped and all tanks were purged of ozonated water. Fish were then maintained with a continuous flow (≃2 l min^{-1}) of clean unfiltered estuarine water for the next 14 days. Two physiological (blood pH; hematocrit) and one histopathological (gill tissue) parameters were monitored during the 18-day exposure-recovery experiment. These parameters were measured on sets of 5 fish, randomly selected from each exposure and control tank following 24, 48 and 96 h of exposure and after 4 and 14 day recovery as described below:

Sample collection. Fish were individually dip-netted at each sample time from the exposure tanks and quickly wrapped in paper towels to facilitate handling. A scalpel was then used to cut the afferent gill artery lying immediately below the right operculum. This procedure caused blood to collect in the shallow cavity produced by the incision and was withdrawn using a heparinized pasteur pipette. Three preheparinized capillary tubes were filled from the pipette and the remaining blood placed in a 3-ml heparinized polyethylene vial. pH was measured immediately using a micro pH electrode (Microelectrodes Inc., Londonderry, NH). The capillary tubes were centrifuged in a microhematocrit centrifuge for 5 min at 2000 *g* and measured using a standard hematocrit reader. The average time from when a fish was netted to when blood collection was completed was approx. 50 s. This sampling technique probably resulted in an underestimation of the absolute blood pH (Garey, 1972), but was utilized since cathetarization of such a large number of fish was impractical. However, relative changes between the control and experimental fish should still be accurate.

After blood sample collection, 3–4 of the anterior gill arches were removed from the right opercular cavity for histological preparation. Whole gill arches were immediately rinsed in an iso-osmotic sucrose solution to remove excess blood. For light microscopy 1–2 gill arches were placed in Davidson's fixative for 96 h. After fixation gills were transferred to 50% ethanol for 24 h and then to 70% ethanol for final dehydration before embedding. Gills were embedded in paraffin, sectioned sagittally and parasagittally at 6–8 μm, stained with hemotoxyline and eosin (H and E) and mounted on glass slides for light microscopy. For scanning electron microscopy (SEM), 1–2 gill arches were fixed in a buffered 4% commercial formaldehyde–1% gluta-

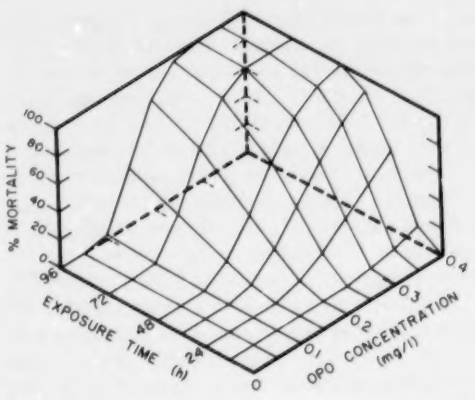


Fig. 1. Mortality response surface for adult white perch (*Morone americana*) exposed to a series of OPO concentrations at 15 C for periods up to 96 h.

raldehyde solution (McDowell & Trump, 1976). Additional SEM preparation and photographic work was performed by the Department of Pathology at the University of Maryland's School of Medicine (Baltimore, MD).

Basic water quality during the exposure and recovery periods was: temperature 15.0 (±0.20) C, salinity 9.0 (±0.50)‰, pH 7.9 (±0.24) and dissolved oxygen concentrations which ranged from 8.7 to 11.7 mg l^{-1} according to the OPO concentration.

Statistical analysis of blood pH and hematocrit was performed using standard ANOVA procedures followed by a Duncan's multiple range test. An arcsine transformation was performed on hematocrit values before analysis since this parameter is expressed as a percentage; pH data were analyzed directly.

RESULTS AND DISCUSSION

Acute toxicity study

The final predictive modeling equation selected by the stepwise analysis for the mortality of adult white perch exposed to OPO at 15 C for 96 h was:

$$Y = 0.2908 - 0.0096(T) + 0.1690(D) - 0.25(D)^2 \quad r^2 = 0.85.$$

Partial *F*-statistics showed the dose (D) to be the single most important variable in this equation. OPO concentration (measured in mg l^{-1}) and exposure time (measured in h) contribute almost equally in producing a given mortality response, although exposure time is slightly more important as indicated by the inclusion of a time variable in the final model. These results are graphically shown in Fig. 1 which was constructed from the modeling equation after back-transforming the predicted response (Y) using the following equation:

$$\text{Predicted percent mortality} = (\sin Y)^2 \times 100.$$

As noted previously, mortality increases similarly along both the time and concentration axis (Fig. 1). This demonstrates the approximately equal contribution made by exposure time and OPO concentration in predicting the mortality of adult white perch.

The 24-, 48- and 96-h LC_{50} concentrations predicted by this model are 0.37, 0.23 and 0.16 mg OPO l^{-1}. Probit regression yielded similar 24-, 48- and 96-h LC_{50}'s (±95% confidence limits) of 0.38 (0.352–0.420), 0.26 (0.236–0.268) and 0.20 (0.181–0.223) mg OPO l^{-1}, respectively. None of the fish exposed to OPO concentrations ≤0.1 mg l^{-1} lost equilibrium or died during the 96-h exposure period. However, most of the white perch exposed to ≃0.1 mg OPO l^{-1} were extremely lethargic and insensitive to tactile stimulation. At concentrations greater than 0.1 mg OPO l^{-1} fish always experienced a period of loss of equilibrium before death.

Little information is available on the toxicity of ozonated estuarine water to fish. However, recent studies on the chemistry of ozonation and chlorination of estuarine and marine waters, which contain bromide, indicate that these two oxidants may produce similar residual oxidant species (Eppley *et al.*, 1976; Helz, 1981; Richardson *et al.*, 1981). Therefore, the toxicity of OPO to adult white perch at 15 C should be similar to that of CPO, assuming similar water quality. This hypothesis is supported when our results are compared with those of Gullans *et al.* (1977) who found the 96-h LC_{50} for adult white perch was 0.21 mg CPO l^{-1} at 15 C. This comparison is justifiable since both our study and Gullans *et al.*'s were conducted at the same laboratory under similar water quality conditions.

Exposure and recovery study

This study was conducted at OPO concentrations near or below the 96-h LC_{50} estimated from the acute toxicity studies. However, the toxicity of the same amperometrically measured OPO concentrations was greater than during the acute study. This toxicity difference was not attributable to any of the standard chemical or physical water quality parameters which were monitored. The resulting loss of fish caused the 0.15 and 0.10 mg OPO l^{-1} concentrations to be terminated after the 48- and 96-h exposure periods, respectively. The control and the remaining fish at the lower concentrations, however, provided an adequate range over which the effects of OPO on both blood chemistry and histopathology could be observed.

Blood pH and hematocrit

ANOVA analysis showed that neither exposure nor recovery time were significant factors in determining blood pH and hematocrit levels. The OPO concentration, however, proved to be highly significant. Therefore, the mean pH and hematocrit values for each exposure concentration were reanalyzed using pooled time data. The results of the multiple range test for both pH and hematocrit are shown in Table 1. In general, blood pH decreased and hematocrit increased with increasing OPO concentration. Blood pH was significantly lower at OPO concentrations

Table 1. Mean responses of blood pH and hematocrit in adult white perch (*Morone americana*) exposed to OPO at 15 C

Blood parameter	OPO concentration (ml l^{-1}) 0.00	0.01	0.05	0.10	0.15
pH	7.47	7.47	7.47	7.31	7.19
Hematocrit (%)	23	23	27	38	39

≥ 0.15 mg l^{-1} while hematocrit increased significantly at concentrations ≥ 0.10 mg OPO l^{-1}. Block *et al.* (1978) also demonstrated that lethal levels of CPO caused a significant reduction in pH with a concomitant rise in hematocrit for adult white perch. Bass & Heath (1977) found similar responses in pH and hematocrit for rainbow trout exposed to CPO. These authors concluded that the changes were the result of internal hypoxia induced by gas exchange blockage at the gill surface. However, the increased hematocrit could also have occurred through swelling of the red blood cells or because of a reduction in plasma volume. Thus the increase in hematocrit may be a physiological compensation to increase oxygen uptake, although further experimentation is needed to determine the exact mechanism. This hypothesis is supported by the work of Skidmore (1970) and Burton *et al.* (1972) who showed that tissue hypoxia was the primary cause of death in rainbow trout whose gill epithelium had been damaged by exposure to zinc sulfate. The reduction in pH may be caused by a buildup of CO_2 and/or lactic acid in the blood and a partial shift to anaerobic metabolism (Bass & Heath, 1977; Wood *et al.*, 1977).

Gill histopathology

Gill tissue samples were extremely useful in clarifying the exact physical mechanism causing mortality of adult white perch exposed to OPO. Unfortunately these effects could not be quantitated morphometrically as discussed by Hughes (1972) and Hughes & Perry (1976). The photomicrographs, therefore, are given only as qualitative examples of the types of gill damage which occurred during the various exposures. Figure 2 shows the progressive tissue destruction caused by increasing OPO concentrations. Plate (A) (Fig. 2) shows a normal gill filament. Slight membrane separations are artifacts produced during tissue slide preparation. Plate (B) was taken from a fish exposed to 0.01 mg OPO l^{-1} for 96 h. At this concentration there is little apparent tissue damage, no physiological changes occurred and fish did not show any abnormal behavior (qualitative observation). Gills from fish exposed to 0.05 mg OPO l^{-1} (plate C), However, exhibited moderate to severe clumping of the secondary lamellae with some excess mucus production and sloughing of epithelial cells. The normal blood pH and slight but insignificant increase in hematocrit shows that although some gill damage has occurred it does not affect gas exchange. Plate (D) shows a dramatic increase in mucus production which has entirely filled the spaces between secondary lamellae of fish exposed to 0.10 mg OPO l^{-1}. Other areas of the gill (not shown) showed severe edema and loss of epithelial cells. The heavy mucus production in the interlamellar spaces undoubtedly restricts gas exchange and the diffusing capacity of the gills as indicated by the significantly higher hematocrit and lower (not significant) pH levels (Table 1). Figure 2(E) and (F) show gills taken from severely impaired fish which had lost equilibrium and were apparently close to death. There is complete disruption of the secondary lamellae, extensive loss of epithelial tissue, and severe hyperplasia (Plate E) and edema. This total destruction always resulted in eventual death of the fish.

The extensive damage to gill epithelium exposed to high OPO concentrations can be clearly seen using scanning electron microscopy (Fig. 3). Plate (A) shows the normal appearance of a gill filament and associated lamellae from a control fish. The squamous epithelial cells are very smooth and give the lamellae a regular appearance. Intercellular boundaries are marked by a relatively inconspicuous double ridge. Plate (B) (from the initial LC_{50} study) shows the effect of 0.3 mg OPO l^{-1} on the epithelium and structural integrity of the secondary lamellae. The secondary lamellae are broken in many areas and have an extremely irregular appearance. Many sites of ruptured or missing epithelial cells can be seen. The squamous epithelial cells which are still intact appear shrunken, making the intercellular boundaries quite pronounced.

Oxidant-damaged gills have been observed previously for freshwater teleosts (Bass & Heath, 1977; Wedemeyer *et al.*, 1979; Paller & Heidinger, 1980) and for an estuarine fish (*Leiostomus xanthurus*) (Middaugh *et al.*, 1977). Little work has been done, however, on the recovery potential of fish surviving moderate to high exposures of OPO or CPO. Bass & Heath (1977) examined the physiological and histopathological responses of rainbow trout exposed to 2-h "pulses" of $\simeq 0.5$ mg CPO l^{-1} every 8 h. They found that the effects of each pulse were cumulative with only minor physiological recovery between the first few pulses. Thus damage done to the gills of rainbow trout requires more than 6 h to repair. MacKinnon & Enesco (1980) have shown that the epithelial cells of teleost fish constitute a cell renewal system. New epithelial cells form near the proximal end of the gill filaments (primary lamellae) and migrate outward toward the distal ends of both primary and secondary lamellae. The migration rate for the rosey barb (*Barbus conchonius*) was approx. 1.2 μm h^{-1} with a projected epithelial replacement rate of about 100 h.

Based upon this replacement rate, severe damage to the gills of white perch should take at least 4 days to

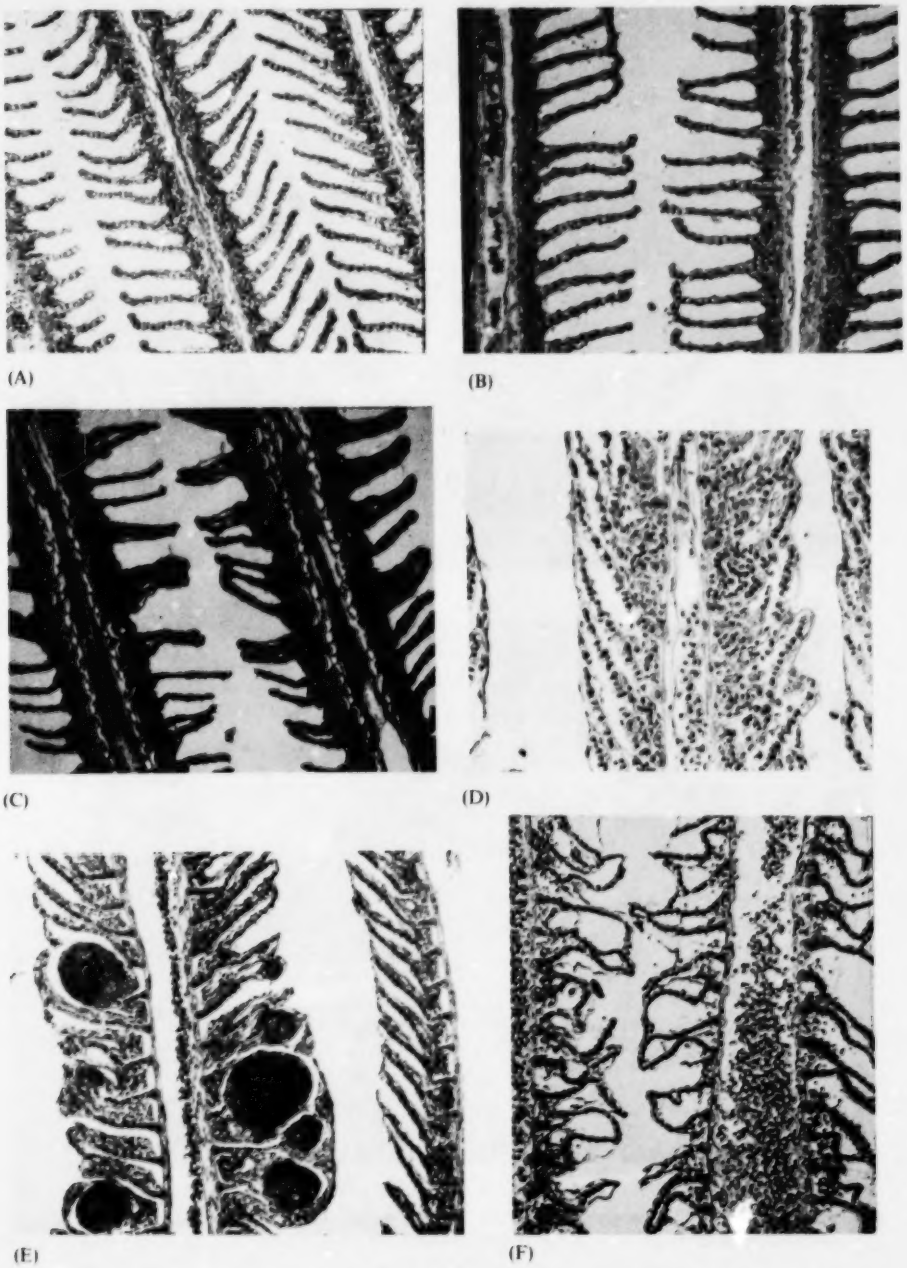


Fig. 2. Sagital sections of the primary and secondary lamellae from white perch (*Morone americana*) exposed to (A) control conditions (× 125); (B) 0.01 mg OPO l^{-1} for 96 h (× 125); (C) 0.05 mg OPO l^{-1} for 96 h (× 125); (D) 0.10 mg OPO l^{-1} for 48 h (× 300); (E) and (F) 0.15 mg OPO l^{-1} for 48 h (× 300).

repair assuming the underlying basal membranes and columnar cells are relatively intact. In our experiments no fish exposed to concentrations ⩾0.10 mg OPO l^{-1} survived through the first 4-day recovery sampling period. Thus was undoubtedly because the gill tissue could not repair itself before restrictions of the respiratory and osmoregulatory systems proved fatal. Gills from fish exposed to 0.05 mg OPO l^{-1} for 96 h, however, showed almost complete recovery within 4 days (Fig. 4A) and complete recovery in 14 days (Fig. 4B). This indicates that the replacement rate in the cell renewal system of the gill epithelium in white perch is of the same order of magnitude as that of the rosey barb (MacKinnon & Enesco, 1980).

(A)

(B)

Fig. 3. Scanning electron micrographs of primary and secondary lamellae from white perch (*Morone americana*) exposed to (A) control conditions (× 1000); (B) 0.3 mg OPO l^{-1} until death (× 500).

CONCLUSIONS

The following conclusions can be drawn from the results of this investigation:

1. The toxicity of OPO to adult white perch is approximately equal to the toxicity of CPO when tested under similar conditions (96-h $LC_{50} \simeq 0.2$ mg l^{-1}).

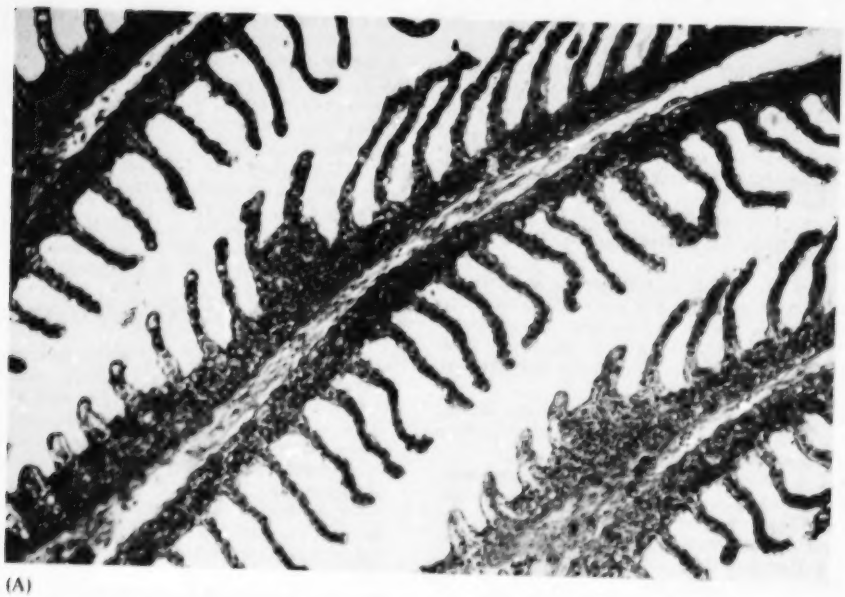
2. OPO causes death by destroying the gill tissue and thus the respiratory and osmoregulatory capabilities of a fish; eventually causing death through internal hypoxia.

3. OPO affects gill tissue in a manner similar to that of CPO.

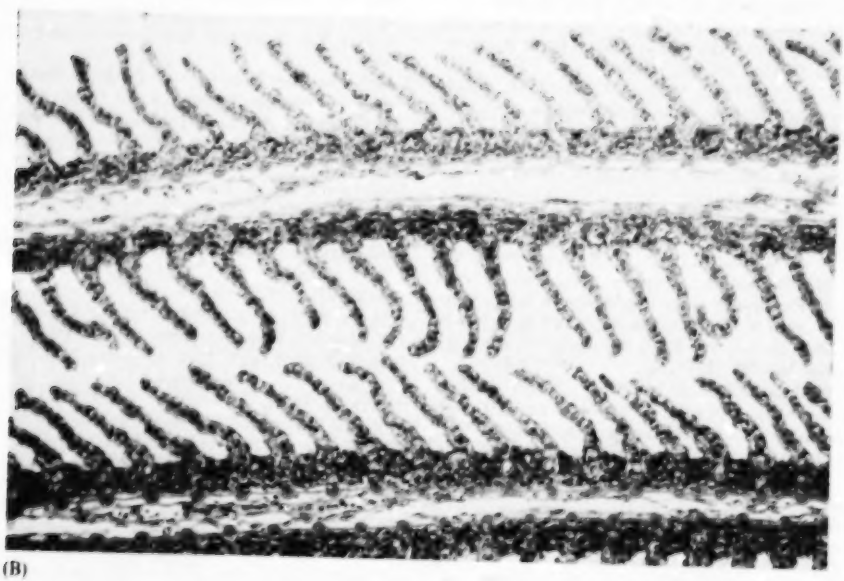
4. Exposure to lethal OPO concentrations causes blood hematocrit to increase and pH levels to decrease.

5. If blood pH has been reduced by more than $\simeq 0.3$ units gill tissue damage is too extensive for recovery and the fish will eventually die.

6. White perch gill epithelium can repair itself within 4 d if damage is moderate (i.e. heavy mucus, minor epithelial cell loss).



(A)



(B)

Fig. 4. Sagital sections of primary and secondary lamellae from adult white perch (*Morone americana*) exposed to 0.05 mg OPO l^{-1} for 96 h and then allowed to "recover" for (A) 4 days; (B) 14 days (A and B × 125).

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